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(71)(72) Applicants and Inventors: SCHACHT, Etienne, Honoré [BE/BE]; Rijsseveldstraat 99, B-8140 Staden (BE). DUNCAN, Ruth [GB/GB]; Department of Biological Sciences, University of Keele, Keele, Staffordshire ST5 5BG (GB). LOCCUFIER, Johan [BE/BE]; Laurent Delveauxstraat 4, B-9000 Ghent (BE).

(74) Agent: H.N. & W.S. SKERRETT; Charles House, 148/9 Great Charles Street, Birmingham B3 3HT (GB).

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(54) Title: CONJUGATE COMPOUNDS OF POLYMERS WITH OTHER ORGANIC MOLECULAR ENTITIES

(57) Abstract

A method is disclosed whereby macromolecular chain polymers can be selectively and covalently coupled to other organic molecular entities to provide conjugate compounds using a reaction between a thiol-specific reactive disulphide group, preferably a sulphenyl thiocarbonate group, in one of the molecular species and a sulphydryl or thiol group in the other molecular species. The method is particularly applicable to the selective coupling of bioactive molecules to polymers that can serve as relatively inert carriers in biological systems, for use for example as drug delivery agents containing site-specific targeting moieties. The method further enables *inter alia* a 1:1 coupling to be achieved between polymeric drug carriers and proteins or polypeptides, including antibodies or antibody fragments.

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CONJUGATE COMPOUNDS OF POLYMERS WITH OTHER ORGANIC MOLECULAR ENTITIES

FIELD OF THE INVENTION

The present invention relates, at least from one aspect, to the production of conjugate compounds by covalent attachment or coupling of organic molecular entities to polymers, especially but not exclusively macromolecules or polymers that can serve as relatively inert carriers in biological systems thereby to be useful for various therapeutic or diagnostic purposes, for example as drug delivery agents.

The invention also relates to the preparation and uses of polymers having one or more thiol-specific reactive groups enabling selective coupling to be achieved to thiol containing compounds, especially bioactive compounds, yielding conjugate products that may have particularly useful applications in biological systems.

BACKGROUND

In relation to the administration of drugs or other relatively low molecular weight bioactive molecules, especially in the field of human medicine or pharmacology, it has already been proposed to use macromolecules, either natural, synthetic or semisynthetic polymers, as carriers for the drug molecules and/or other molecular entities involved. For example, in the particular case of drug molecules, these may be coupled to the macromolecular polymer to form a polymer/drug conjugate by covalent biodegradable bonds designed to permit a controlled release of the drug within the body of the recipient, such release in some instances occurring actually within particular cells that are able to take up the polymer/drug conjugate. Moreover, at least with a soluble biologically inert

macromolecular polymer carrier capable of becoming distributed or circulating within the body, there is a possibility of also attaching or coupling to the polymeric carrier other residues or molecular entities, termed targeting moieties or determinants, capable of recognising and interacting with specific sites or cell surface receptors whereby the polymeric drug carrier may be "targeted" to the particular areas or cells where the drug is required before the drug is released. Thus, such targeting moieties or determinants, which can include molecular entities such as hormones, antibodies or other proteins, can permit site-specific drug delivery. Once at the target location, drug release then takes place by biodegradation and cleavage of the bonds linking or coupling the drug molecules to the carrier, e.g. by hydrolytic cleavage promoted by intracellular enzyme systems following pinocytic uptake of the carrier/drug conjugate.

The design and uses of such drug delivery systems is already quite well documented in the literature. For example, a review by J. Kopecek and R. Duncan of systems employing synthetic polymer carriers based on poly[N-(2-hydroxypropyl)methacrylamide] (polyHPMA) is to be found in the Journal of Controlled Release, 6 (1987), 315-327, and at least certain features of such synthetic polymeric drugs are disclosed, inter alia, in EP 0187547. Other synthetic or semisynthetic polymers that have been proposed for use as macromolecular drug carriers include polyamino acids and polysaccharides like dextrans, and the range of natural polymers that have been considered includes macromolecules such as proteins, although in some cases small proteins (e.g. antibodies, enzymes and hormones) may even be the molecular entities which it is desired to couple to another carrier polymer.

In summary, macromolecular drug carriers can be regarded as affording a possibility of restricting the body

distribution, and of controlling the release, of drugs thereby to improve their therapeutic index. However, for successful practical applications it is essential to identify and utilise drug/polymer linkages that are capable of controlled biodegradation, and in order to optimise the activity of such macromolecular drug carriers residues capable of acting as targeting moieties or determinants need to be incorporated into their structure as previously referred to. For this purpose, it is accordingly important to produce well-defined macromolecular conjugates which, ideally, should contain a high payload of drug molecules but only a single targeting group is required, i.e. a 1:1 coupling between the polymer carrier and the targeting group.

In any event, it will be appreciated that in this particular field at least, there is a need to couple to macromolecular polymeric carriers, by suitable degradable covalent bonds, drug molecules and also other bioactive molecular entities adapted to act for example as targeting moieties, and this is an area in which the present invention can be especially useful.

As indicated, the molecules to be coupled to the polymeric carrier may include proteins of which antibodies (or antibody fragments) provide one example. The concept of using antibodies, especially monoclonal antibodies, for therapeutic purposes and as targeting moieties in conjunction with drugs, coupled either directly or by way of an intermediary macromolecular drug carrier, is in fact quite attractive having regard to the high specificity of antibodies. However, considerable difficulties have hitherto been encountered in devising practical systems for use in human medicine, due for example to interference with the antigen binding sites and loss of antibody specificity following coupling to another molecule, adverse immunological response effects, and problems arising in relation to transport to the required cellular or tissue

location. The latter difficulty can be particularly acute if the antibody conjugate is too large for efficient uptake from the bloodstream. Although antibody complexes, e.g. antitumour antibodies conjugated to radioisotopes, have been used and administered in human medicine for diagnostic applications, in which case the fact that only a very small percentage of the antibody conjugates may actually reach the relevant location can often be compensated for by sophisticated computer-imaging techniques, for therapeutic applications it is essential that a high concentration of the antibody conjugates should reach the relevant location. For this reason at least, the size of the coupled molecular entities in any carrier polymer/antibody conjugates needs be kept as small as possible, and generally it may be advantageous to use only active antigen binding fragments of the antibody proteins for targeting purposes.

For a discussion of some of the problems and future prospects concerning the use in human therapy of antibodies, particularly monoclonal antibodies, reference may be made to a review article by F.M. Brodsky in Pharmaceutical Research, Vol. 5, No. 1 (1988), 1-9, and a discussion relating more specifically to the conjugation of monoclonal antibodies with cytostatic agents or toxins is to be found in an article (pp 81-85) by Sedlacek et al (1985) entitled "Monoclonal Antibodies in Tumour Therapy" in Vol. 32 of the publication "Contributions to Oncology" (Ed. S. Eckhardt et al) published by Karger.

Many attempts have been made to develop antibody-polymer systems in order to improve drug delivery and, possibly in some cases, to reduce immunogenic effects of antibodies when administered to a "foreign" host. Several well-documented methods are available for conjugation. For coupling HPMA copolymers to antibodies (or other proteins) for example, one method has recently been disclosed by Flanagan et al (Biochimica et Biophysica Acta, 993 (1989) 83-9) in which reactive nitrophenol ester-linked groups

(–ONp) within the HPMA copolymer are reacted by means of aminolysis with $-NH_2$ groups within amino acid sequences of the antibody. However, it is believed that none of these known methods has satisfactorily resolved the problem of achieving 1:1 coupling of protein:polymer in a precisely definable manner, and in general there still remains a need for improved methods for carrying out selective coupling of antibodies or antibody fragments, and of other proteins, to macromolecular polymeric carriers.

SUMMARY OF THE INVENTION

In one specific aspect, the present invention can provide a solution to this problem, as well as providing a means of achieving selective coupling between other polymers or between polymers and various other organic molecular entities which, particularly in the case of bioactive molecules, may be useful in biological systems.

The invention is based broadly on the application of a concept of covalently coupling together a macromolecular chain polymer compound and another organic molecular entity utilising a method in which a thiol-specific reactive disulphide or sulphenyl thiocarbonate group, –S–S–CO–OR where R is alkyl, aryl or alkylaryl, in one of the molecular species is reacted with a sulphydryl or thiol group present or provided in the other molecular species.

In carrying out the invention it is preferred to provide thiol-specific reactive sulphenyl thiocarbonate groups as specified above, for example methoxycarbonyl-disulphide groups, since these react easily and rapidly with sulphydryl or thiol (–SH) groups (hereinafter termed simply thiol groups) resulting in fragmentation yielding carbonyl sulphide and an unsymmetrical disulphide, as first reported by Brois et al (J.A.C.S., 92 (1970), 7629). This reaction, it has been found, can be carried out in aqueous media e.g. phosphate buffer, and under mild conditions

without application of heat, e.g. at room temperature or below. At the same time, however, such sulphenyl thiocarbonate groups are generally stable to many other functional groups and are thus highly specific in their reaction with thiols.

However, in at least some cases it can be possible as an alternative to provide other reactive disulphide groups that can react in a similar manner with thiol groups.

More specifically, in one aspect, the invention provides a process for preparing a conjugate compound in which a macromolecular chain polymer is selectively coupled to another organic molecular entity, said process being characterised by the steps of:

(a) modifying or forming the polymer so as to provide it with a thiol or protected thiol group in a predetermined position or positions,

(b) providing a derivative of said other molecular entity containing a thiol or protected thiol group,

(c) treating the compound of either step (a) or step (b) to convert the thiol or protected thiol group thereof into a thiol-specific reactive disulphide or sulphenyl thiocarbonate group, —S—S—CO—OR where R is alkyl, aryl or alkylaryl, and

(d) reacting together the compound of step (a) or step (b) that is not utilised in step (c) with the product of step (c) whereby the thiol-specific reactive disulphide or sulphenyl thiocarbonate group of the latter reacts with the thiol or protected thiol group of the other reactant to result in the formation of an unsymmetrical disulphide covalent linkage between the polymer and said other molecular entity.

The macromolecular chain polymers can have a wide variety of molecular weights, ranging for example from several hundred to many tens of thousands of daltons. For use in biological systems, they will usually be water soluble polymers.

In practice, to provide the preferred reactive sulphenyl thiocarbonate group, this is conveniently formed by reacting a thiol or (preferably) a protected thiol group, i.e. –SY where Y is hydrogen or a protective group such as trityl, tert. butyl or acetamidomethyl, with a carbonyl sulphenyl halide, Hal–S–CO–OR, for example an alkoxycarbonylsulphenyl chloride (carboalkoxy-sulphenyl chloride). Insofar as the molecular species concerned does not initially contain the thiol or protected thiol group –SY which is required for reacting with the carbonyl sulphenyl halide, this is introduced, e.g. by way of an aminolysis reaction using a thiol amine (mercaptoamine derivative) as a carrier, in a preliminary stage of the process.

Alternative thiol-specific reactive disulphide groups as previously referred to may be formed by reacting a thiol or protected thiol group with an aromatic sulphenyl halide such as 2-nitrophenyl sulphenyl chloride or 2-pyridyl sulphenyl chloride for example.

The molecular species provided or formed with the reactive disulphide or sulphenyl thiocarbonate group is preferably the polymer to which the other organic molecular entity is to be coupled. A multi-functional polymer containing a plurality of thiol-specific reactive groups can be formed by the same basic reaction strategy and may then be used selectively to couple a plurality of other organic molecular entities at different locations, generally through spacer linkages and disulphide bonds. The invention may very advantageously be applied for example to the coupling of drug molecules (especially –SH

containing drugs such as the antitumour drugs mercaptopurine and thioguanine for example) and/or targeting moieties (especially antibodies or antibody fragments and other small thiol-containing proteins) to soluble polymers which provide macromolecular carriers for controlled drug delivery. In the case of the attachment or coupling of drug molecules by this method to the polymer carrier, the disulphide bonds generated between the drug and carrier will generally be biogradable and susceptible to cleavage for drug release, either extracellularly (e.g. by thiols such as cysteine or glutathione) or intracellularly inside the lysosomal compartment of a cell following partial proteolysis where the drug is in the nature of a protein (e.g. the antileukaemic drug asparaginase).

Multi-functional polymers containing a plurality of the thiol-specific reactive groups as referred to above may, of course, also contain other reactive groups, e.g. reactive esters or reactive carbonates that allow coupling with amines, which can also be utilised for attaching other drug molecules or molecular entities without use of a disulphide bond. Or, especially for a polymer which does have, or is intended to have, a plurality of such other reactive groups for attaching drug molecules, the polymer may be provided or formed with only one of the thiol-specific reactive groups, e.g. for coupling a single targeting moiety or protein such as an antibody fragment. Such single thiol-specific reactive group may be located along the length of the polymer or, more preferably, as a functional terminal group at an end of the polymer chain, e.g. in the case of poly-α-aminoacids or monomethoxy-polyethylene glycols. Alternatively, one of the thiol-specific reactive groups may be formed or provided at each terminal chain end of the polymer, as for example in the case of an α,Ω-bifunctional polyethylene glycol or polyethylene oxide, so that the polymer only has functional thiol-specific reactive end groups.

It will be appreciated that providing the polymer with only a single thiol-specific reactive group, preferably as an end terminal group, affords the possibility of coupling thereto in a well-defined and predictable controllable manner just one thiol-containing protein molecule. Since many proteins, especially Fab antibody fragments, may contain only a single thiol group, this therefore provides a route to achieving a 1:1 polymer/protein conjugate which hitherto has been a result that has proved elusive to many researchers in this field.

Thus, the invention also provides a method of selectively coupling a thiol-containing protein or polypeptide to a macromolecular polymer carrier wherein the polymer carrier is provided or formed with a single thiol-specific reactive disulphide or sulphenyl thiocarbonate group and is then reacted with the protein or polypeptide so as to result in interaction with a thiol group in the latter and establishment of a covalent disulphide linkage between the protein or polypeptide and the macromolecular polymer carrier to form a conjugate compound thereof.

The invention also provides conjugates of a macromolecular polymer carrier and a protein or polypeptide in which covalent coupling is established by a covalent disulphide linkage. The invention further provides such conjugates in which there is a polymer to protein or polypeptide conjugation ratio of 1:1.

As previously indicated, in the preferred strategy for providing or forming the polymers with the thiol-specific reactive disulphide or sulphenyl thiocarbonate group or groups, the polymers are initially prepared with one or more protected –SH substituents which, possibly after performing other reactions to modify the polymer and attach other drug molecules for example, are finally activated and converted to the corresponding reactive disulphide groups, preferably sulphenyl thiocarbonate groups, by treatment

with a sulphenyl halide immediately prior to coupling with an -SH containing protein, antibody or antibody fragment, or other organic molecular entity. Preferred -SH protective groups include trityl, tert, butyl and acetamidomethyl groups which are readily removed in the reaction with the sulphenyl halide reagent, but any other suitable protective groups which are likewise readily removed by the sulphenyl halide reagent could equally well be used instead. With regard to trityl and acetamidomethyl protective groups, it may be noted that the former is rather hydrophobic whereas the latter is much more hydrophilic, and since this can affect the solubility properties of the conjugates formed this may be a factor that will influence the choice of protective group to be used in practice.

The use of the protected thiol group which is activated only in the later stage is in fact a most important aspect in the preferred strategy. By this means the carrier polymer can first be prepared with a protected thiol group and, for example, with OH groups. The latter can then be (partially) converted into reactive derivatives (e.g. carbonate esters) which allow coupling with drug moieties. Only in the last stage is the thiol group activated and coupled with another thiol-containing molecule, such as a thiol-containing protein, e.g. Fab'-SH. This scheme permits the conjugates to be prepared in organic media up to the point where they are to be coupled with the other molecular entity which, in the case of a protein for example, may be quite fragile. This coupling reaction, however, can be carried out in aqueous buffer and under mild conditions so that risks of degradation at this stage are minimized. With a protein, for example, if coupling should occur in an earlier stage it may be damaged or degraded during subsequent chemical modification of the polymeric carrier.

In connection with the preparation of macromolecular

structures for use as macromolecular prodrugs, the invention therefore enables polymers to be prepared carrying different moieties that can be introduced stepwise and under acceptable reaction conditions.

When using a carbonyl sulphenyl halide reagent, most commonly methoxycarbonyl sulphenyl chloride will be a preferred choice on account of its widespread availability and convenient handling characteristics, but other halides such as bromides could also be used. In any event, the carbonyl sulphenyl halide will usually be an alkoxycarbonyl halide with the alkyl group being methyl, ethyl, propyl, isopropyl or butyl since the halides, or at least the chlorides, of these alkoxycarbonyl sulphenyl reagents are most readily capable of purification by distillation.

In dealing with an existing polymer which does not initially contain any sulphide or thiol group the required thiol or protected thiol group or groups must be introduced in a preliminary stage as already mentioned, and for this purpose the polymer may need first to be converted into a reactive derivative that can link with an amine or other carrier used to introduce the thiol or protected thiol group(s).

Thus, in the case of an existing —OH containing polymer such as dextran for example, one or more of the —OH groups along the length of the chain may first be activated by reacting with p-nitrophenyl chloroformate in DMSO (dimethylsulphoxide)/pyridine at 5°C to form the p-nitrophenyl carbonate —O—CO—ONp (ONp = p-nitrophenoxy) or cyclic carbonate derivative which reacts smoothly with amines (aminolysis) with formation of a corresponding urethane derivative. The number of such reactive groups formed along the dextran chain can be controlled by the amount of chloroformate added and by the reaction conditions such that any activation percentage between 0 and 30% of the anhydro glucoside units in the dextran chain

can be achieved.

Having formed the requisite number of reactive ONp or carbonate groups, the polymer may then be reacted with mercapto-ethylamine or, for example, 2-S-trityl mercapto-ethylamine, to form the required number of thiol or protected thiol groups, whilst other remaining reactive ONp or carbonate groups, if any, may be coupled in known manner by aminolysis (in organic media) to other molecular entities or compounds containing aliphatic $-NH_2$ groups and drug molecules. The polymer can then be treated with alkoxycarbonyl sulphenyl chloride to activate the thiol or protected thiol group(s), producing the corresponding thiol-specific reactive sulphenyl thiocarbonate derivative, following which reaction (in aqueous media) with another -SH containing compound, especially a -SH containing protein, polypeptide or other bioactive molecule adapted to act as a targeting moiety, establishes a covalent coupling between the latter and the polymer.

In a particular example performed to demonstrate the general feasability of this method for coupling -SH containing compounds to dextran, 2-(S-trityl)mercapto-ethylamine was reacted with chloroformate-activated dextran (3 arom. carb./100 glucose units). The S-trityl substituted dextran was subsequently treated with methoxycarbonyl sulphenyl chloride. The resulting methoxycarbonyl disulphide derivative was isolated via precipitation and subsequent preparative gel filtration. NMR analysis confirmed the anticipated structure. The reactivity of this activated dextran disulphide derivative was evaluated using isopropyl thiol and reduced glutathione as model thiol-containing compounds. H-NMR and amino acid analysis indicated rapid and quantitative formation of the desired disulphide. This method could therefore be used likewise for the selective coupling of Fab-fragments, via their α-specific thiol groups (formed on fragmentation in known manner of the parent antibody), to polymer carriers

such as dextran which can also be linked or coupled, generally via biodegradable spacers such as oligopeptides, to various kinds of drug molecules.

The main reactions referred to above for coupling a Fab antibody fragment to an –OH containing polymer Pol–OH such as dextran are outlined below:

$HS{-}CH_2CH_2{-}NH_2$ $\downarrow$ $\quad\quad$ Pol–OH $\xrightarrow{\text{+ p-nitrophenyl chloroformate}}$

$Trityl{-}S{-}CH_2CH_2{-}NH_2$ + activated Pol–OH

$\downarrow$

$Pol{-}O{-}CO{-}NH{-}CH_2CH_2{-}S{-}Trityl$

$\downarrow$ + Cl–S–CO–OMe

$Pol{-}O{-}CO{-}NH{-}CH_2CH_2{-}S{-}S{-}CO{-}OMe$

$\downarrow$ + HS–Fab

$Pol{-}O{-}CO{-}NH{-}CH_2CH_2{-}S{-}S{-}Fab$ + MeOH + S=C=O

In applying the invention to provide terminally modified polymers with functional thiol-specific reactive disulphide groups such as sulphenyl thiocarbonate end groups for coupling an end or ends of the polymer chain to another –SH containing residue, where the polymer initially has a hydroxyl or similar functional end group or groups, as with poly(ethylene glycol) (PEG) or polyethylene oxide for example, the same general scheme as has been described above for dextran may be followed, i.e. the end group or groups may first be activated by treatment with an activating agent such as p-nitrophenylchloroformate and is then reacted with a protected thiol-containing primary amine such as 2-[S-trityl]mercaptoethylamine to provide a protected thiol derivative that is subsequently activated

and converted into a thiol-specific reactive group by treatment with methoxycarbonyl sulphenyl chloride to yield the corresponding methoxycarbonyl disulphide or sulphenyl thiocarbonate derivative. This can then be isolated and reacted as required with the –SH containing residue to be coupled.

The potential provided by the present invention for producing well-defined conjugates of proteins with biologically inert polymers such as polyethylene glycol (PEG), e.g monomethoxypolyethyleneglycol, having only a functional end group is of especial interest since there is some evidence to suggest that polymers of this kind can reduce the immunogenicity of "foreign" proteins and can increase their stability. This can be of particular importance in connection with administration of enzymes used as drugs, e.g. the antileukaemic drug asparaginase, which otherwise normally have only a short active lifetime after injection and which can also cause an adverse immune response after repeated administration, as well as in connection with proteins such as monoclonal antibodies used for targeting which likewise are likely to cause an adverse immune response after repeated administration. Thus, by coupling one or more polymers such as polyethylene glycol (or polyethylene oxide), provided with a thiol-specific reactive disulphide or sulphenyl thiocarbonate group in accordance with the invention, to such proteins via thiol groups in the latter, the immunogenicity of the proteins may be reduced and their stability increased, thereby significantly improving the possibilities for more effective therapeutic use. Moreover, other advantages may also arise with such conjugates, for example improvements in the hydrophilic/hydrophobic characteristics of the proteins, depending on the molecular size and properties of the polymer used.

The invention also relates to the production of polymers such as polyamino acids having a terminal thiol-

specific reactive group at one end, and to the coupling thereof to any suitable protein or other molecular species, especially a bioactive molecular species, having a thiol group, again with the possibility of producing 1:1 conjugates. The invention further provides a novel method for synthesis of such polymers, especially polyamino acids, that may also include a plurality of other functional groups, e.g. hydroxyl groups on side chains or spacers, available for coupling to other molecular entities, such as drugs, along the length of the polymer chain.

This novel method of synthesis is based on the use of a mercaptoamine derivative, especially an —SH protected mercaptoamine YS—R—NH_2 or an active derivative thereof, as an initiator for the polymerisation of monomer units, for example N—carboxyanhydride (NCA) derivatives of aminoacids, whereby the mercaptoamine derivative becomes incorporated as an end unit of the polymer chain. It therefore serves not only as a polymerisation initiating agent but it also serves to introduce the thiol or protected thiol group for subsequent activation, e.g. by treatment with an alkoxycarbonyl sulphenyl halide, or to introduce the thiol-specific reactive disulphide or sulphenyl thiocarbonate group directly.

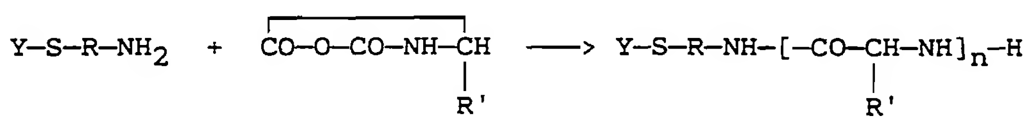
Thus, from this further aspect the invention also provides a process for preparing a conjugate compound in which a synthetic macromolecular chain polymer is selectively coupled to another organic molecular entity, characterised in that during the process

(a) the polymer is synthesised from monomer units in a polymerisation reaction initiated by a mercaptoamine derivative that becomes incorporated as an end unit of the polymer chain;

(b) the polymer is formed with a thiol-specific reactive disulphide or sulphenyl thiocarbonate group, —S—S—CO—OR where R is alkyl, aryl or alkylaryl, in said end unit of the polymer chain;

(c) the polymer containing said thiol-specific reactive disulphide or sulphenyl thiocarbonate group is reacted with a thiol group containing derivative of said other molecular entity whereby the thiol-specific reactive disulphide or sulphenyl thiocarbonate group of the polymer reacts with the thiol group of the other reactant resulting in the formation of an unsymmetrical disulphide covalent linkage between the polymer and said other molecular entity.

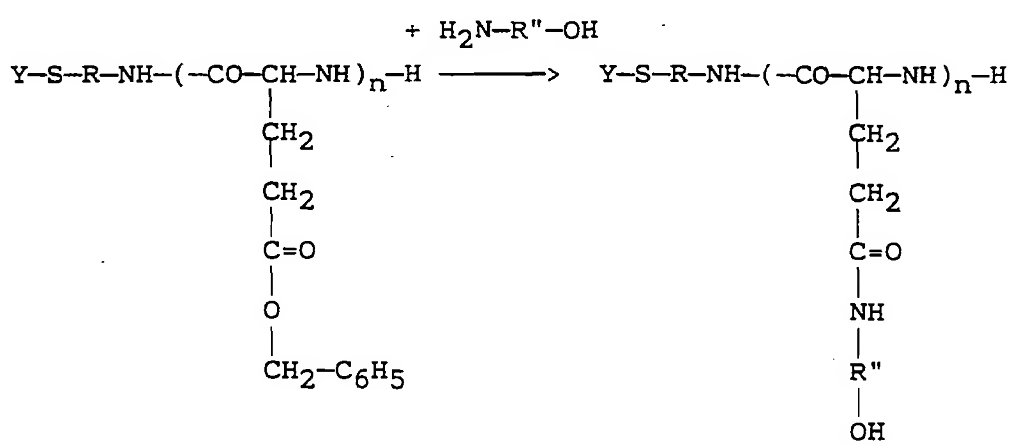
A typical scheme for this synthesis, applied to a polyamino acid and using an amino acid NCA derivative or derivatives, may be depicted as follows:



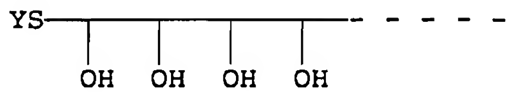
where, for example, Y = trityl or acetamidomethyl

and R' = $-CH_2CH_2-CO-O-CH_2-C_6H_5$

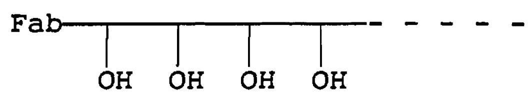
In a subsequent step the benzylester used as a $-COOH$ protective group in the side chain is transformed into an amide by reaction with a hydroxylamine $H_2N-R''-OH$, e.g.



A structure is thereby obtained which may be represented as:



After, activation (e.g. methoxycarbonyl sulphenyl chloride) it may then be coupled via a thiol group to an antibody fragment HS—Fab to provide a protein conjugate:



Derivatives of the thiol-protected mercaptoamine may also be used as the —SY introducing agent and polymerisation initiator, e.g. Trityl-S—R—NH—CO-$(CH_2)_n$—NH_2. Also, if desired, the —SY group could even be activated to produce the thiol reactive disulphide or sulphenyl thiocarbonate group in the amine compound prior to using the latter as an initiator for the NCA polymerisation, although in practice this will often not be desirable since the alkoxycarbonyl disulphide or sulphenylalkoxy thiocarbonate group may become cleaved during subsequent treatment of the polymer before reaching the stage at which coupling is required. Therefore, activation of the protected thiol group by treatment with an alkoxycarbonylsulphenyl halide will usually be delayed until a later stage, shortly before the coupling reaction is to be performed, in the normally preferred procedure.

It will also be appreciated that since many proteins and peptides which it may be desired to couple to a polymer carrier as herein described can now be genetically engineered or synthesised, they may be specifically "tailored" to contain appropriate aminoacids, e.g. thiol-containing aminoacids, to facilitate the coupling and this may also be of assistance in establishing a close control of the conjugation ratio and location of the linkages to the polymer. It is, for example, easily possible in some

cases to ensure that the protein or peptide to be coupled contains only one thiol group, thereby facilitating the production of a 1:1 conjugation ratio.

The invention also includes pharmaceutical formulations comprising polymer conjugate compounds as herein disclosed, especially conjugate compounds containing cytotoxic and/or other bioactive drug molecules and a "targeting" moiety, made up for administration in any suitable manner, for example parenterally (including intravenously, intramuscularly and subcutaneously) or orally. Such formulations, containing for example therapeutically effective amounts or dosages of the polymer/drug conjugates together possibly with at least one other ingredient providing a compatible pharmaceutically acceptable additive, diluent or excipient, may be prepared by any of the methods well known in the art of pharmacy.

DETAILED DESCRIPTION

By way of further background explanation and description of the invention, more detailed examples are hereinafter presented of some experiments made to prepare or form polymers with a thiol-specific reactive group or groups suitable for coupling to –SH containing compounds in accordance with the invention, and of the preparation of "model" conjugate compounds using such polymers.

EXAMPLE 1

Preparation of a monomethoxypolyethyleneglycol (polyether) polymer with a terminal thiol-specific reactive group; $CH_3-[CH_2CH_2-O]-CH_2CH_2-O-CO-NH-CH_2CH_2-S-S-CO-Me$ and coupling to "model" thiol-containing compounds

a) 2-S-trityl mercapto ethylamine (1)

3g (26.5 mmol) 2-aminoethanethiol and 7g (26.9 mmol) trityl alcohol were dissolved in 20ml trifluoro acetic acid. The reaction was allowed to continue for 20 minutes. The solvent was removed under reduced pressure at 60°C. The oily residue was treated with 400ml ether and stored overnight at −18°C to allow crystallization. The product was isolated by filtration, washed with ether and water at 0°C, and dried under high vacuum. The isolated product was suspended in 100ml water. 30ml of a concentrated ammonia solution was added and the aqueous phase was extracted 4 times with 100ml ether. The pooled ether phase was dried over $MgSO_4$, the solvent was then removed under reduced pressure and again the product was dried under high vacuum.

^1H NMR spectrum (500 MHz, solvent: MeOD d_3):

methylene function α of the protected thiol: δ=2.33 ppm, m=3 (2H);

methylene function α of the ammonium: δ=2.41 ppm, m=3 (2H);

para protons of the trityl group: δ=7.21ppm, m=3(3H);

metaprotons of the trityl group: δ=7.28 ppm, m=3(6H);

orthoprotons of the trityl group: δ=7.41 ppm, m=2(6H).

b) <u>α-methoxy-Ω-2-(S-trityl)mercaptoethylamino carbamoyl poly(oxyethylene) (2)</u>

1g (1.33 mmol) poly(oxyethylene) was dissolved in 10ml dry CH_2Cl_2 and 4ml dry pyridine. The mixture was cooled to 0°C. 1g (4.96 mmol) 4-nitrophenyl chloroformate and 72 mg (0.59 mmol) DMAP (72 mg) were added. The mixture was stirred under argon for 4 hours at 0°C. 2g (6.5 mmol) 2-S-(trityl)mercapto-ethylamine was added. The reaction was allowed to continue at room temperature for 48 hours and the solvent was then removed under reduced pressure. The residue was dissolved in water and extracted with 50ml ether. The polymer was isolated by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

^1H NMR spectrum (360 MHz, solvent: MeOD d_3):

methylene function α of the S-trityl group: δ=2.32 ppm, m=3 (2H);

methylene function α of the urethane (CH_2-NH): δ=2.97, m=3 (2H);

methoxy group of the polyether: δ=3.33 ppm, m=1 (3H); the methylene functions of the polyether: δ-3.65 ppm, broadened (50 H);

methylene function α of the urethane (CH_2-O): δ=4.11 ppm, m=3 (2H):

ortho protons of the trityl group: δ=7.36 ppm, m=2 (6H);

meta protons of the trityl group: δ=7.27 ppm, m=3 (6H);

para protons of the trityl group: δ=7.21 ppm, m=3 (3H)

IR spectrum (film):

C-O-C stretching vibration: $v=1100cm^{-1}$;

stretching vibration of urethane: $v=1710cm^{-1}$;

C-H vibrations: $v=2860cm^{-1}$;

N-H vibration of the urethane: $v=3400cm^{-1}$

c) <u>α-methoxy-Ω-2-(S-methoxythiocarbonyl)mercaptoethyl-amino carbamoylpoly(oxyethylene) (3)</u>

100mg (91 μmol) of the S-trityl terminated poly-(oxyethylene) (product 2) was dissolved in 1ml deuterated methanol and 1ml chloroform. 12.3μl methoxycarbonyl sulphenyl chloride (0.135 mmol) was added. The reaction was followed by NMR spectroscopy (360 MHz). After 4 hours, the solvent was removed under decreased pressure. The polymer was isolated by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

^1H NMR (500 MHz, solvent D_2O):

methylene protons α of the sulphenyl thiocarbonate: δ=2.96 ppm, m=3 (2H);
methoxy group of the polyether: δ=3.39 ppm, m=1 (3H);
methylene protons α of the urethane (CH_2—NH): δ=3.43 ppm, m=3 (2H);
methylene protons of the polyether: δ=3.71 ppm, broadened (48H);
methyl group of the sulphenyl thiocarbonate: δ=3.92 ppm, m=1 (3H);
methylene protons α of the urethane (CH_2—O): δ=4.22 ppm, broadened (2H)

d) <u>α-methoxy-Ω-2-(2-aminoethyl)dithioethylamino carbamoyl poly(oxyethylene) (4)</u>

60mg (64 μmol) sulphenyl thiocarbonate terminated poly(oxyethylene) (product 3) was dissolved in 2ml D_2O. A solution was made of 28.6mg thioethanol amine hydrochloride (0.25 mmol) in 1ml D_2O, this compound being selected as a "model" thiol-containing compound. 250μl of this solution was added to the polymer solution. After 2 hours a sample of 1ml was withdrawn, and analysed by ^1H NMR spectroscopy (360

MHz). A second sample of 1ml was analysed by ^{1}H NMR spectroscopy after 24 hours. Both fractions were pooled and freeze dried. The coupled polymer was isolated by preparative GPC on a Sephadex G15 column (eluent: double distilled water), followed by freeze drying.

^{1}H NMR spectrum (360 MHz, solvent D_2O):

NH–$\underline{CH_2}$–CH_2–S–S– : δ=3.48 ppm, m=3 (2H);
NH–CH_2–$\underline{CH_2}$–S–S– : δ=2.88 ppm, m=3 (2H);
–S–CH_2–$\underline{CH_2}$–NH_3+ : δ=3.41 ppm, m=3 (2H);
–S–S–$\underline{CH_2}$–CH_2–NH_3+ : δ=3.00 ppm, m=3 (2H);
methoxy group of the polyether: δ=3.41 ppm, m=1 (3H);
methylene functions of the polyether: δ=3.70 ppm, broadened (67H);
methylene function α of the urethane (CH_2–O): δ=4.23 ppm, broadened (2H)

e) <u>α–methoxy–Ω–2–(glutathionyldithio)ethylamino carbamoyl poly(oxyethylene)</u>

A second example of a "model" thiol-containing compound for coupling to the polymer was provided by reduced glutathione. Glutathione is a tripeptide widely occurring in biological systems as an intra-cellular non-protein thiol which is believed to play an important role in protecting cells from damage by toxic substances, and it has already been proposed to provide conjugates of glutathione with soluble macromolecular carrier polymers such as dextran for use in therapeutic applications (see for example Y. Kaneo <u>et</u> <u>al</u>. <u>Pharmaceutical Research</u> (1989) Vol. 6, No. 12, pp 1025-1031, published by Plenum Publishing Corporation).

60mg (54 µmol) sulphenyl thiocarbonate terminated poly(oxyethylene) (product 3) was dissolved in 2ml

oxygen free phosphate buffer (pH=7, 0.1M). A solution containing 23mg reduced glutathione (75 µmol) was added and allowed to react for 1 hour at room temperature. The coupling product was isolated by preparative gel filtration over a Sephadex G-15 column (eluent distilled water) followed by freeze drying. A 1:1 coupling was evidenced by reducing the reaction product with dithiotreitol and HPLC and capillary electrophoresis analysis of the products formed. NMR analysis further substantiated the proposed structure.

1H NMR (D_2O):	<u>CH3</u>—O—PEO	δ = 3.39 ppm
	S—S—<u>CH2</u>	δ = 2.95 ppm
	NH—CO—<u>CH2</u> (glutamine)	δ = 2.18 ppm
	NH—CO—CH2<u>CH2</u> (glutamine)	δ = 2.52 ppm

EXAMPLE 2

Preparation and coupling of a dextran carrying $-O-CO-NHCH_2CH_2-S-S-CO-OCH_3$ and amine $-O-CO-NHCH_2CH(OH)CH_3$

Materials and Methods

All products were used without further purification. DMSO and pyridine were dried over CaH_2, methanol was dried over CaO and Mg, and formamide was dried over Na_2SO_4.

^1H NMR spectra were recorded on a Bruker WH360 and a Bruker WH500 spectrometer. Preparative size exclusion chromatography was carried out on Sephadex G15 (Pharmacia; Sweden). Double distilled water was used as eluent (2.5ml/min) and eluting components were detected via refractive index.

a) 2-S-trityl mercapto ethylamine (1)

As in Example 1, 3g (26.5 mmol) 2-aminoethanethiol and 7g (26.9 mmol) trityl alcohol were dissolved in 20ml

trifluoro-acetic acid. The reaction was allowed to continue for 20 minutes. The solvent was removed under reduced pressure at 60°C. The oily residue was treated with 400ml ether and stored overnight at −18°C to allow crystallization. The product was isolated by filtration, washed with ether and water at 0°C and dried under high vacuum. The isolated product was suspended in 100ml water. 30ml of a concentrated ammonia solution was added and the aqueous phase was extracted 4 times with 100ml ether. The pooled ether phase was dried over $MgSO_4$. The solvent was removed under reduced pressure and the product was dried under high vacuum.

b) <u>2-S-trityl mercapto ethylaminocarbamoyl dextran (2)</u>

2g dextran (12.3 mmol anhydro glucose units) was dissolved in 60ml dry DMSO and 60ml dry pyridine and was cooled to 0°C. 0.2g (1 mmol) 4-nitrophenyl chloroformate and 12mg (0.1 mmol) 4-dimethylamino pyridine was added. The reaction was allowed to continue at 0°C for 4 hours. The polymer was precipitated in 250ml ethanol, 125ml acetone and 125ml ether, and was intensively washed with ethanol and ether. The product was isolated by filtration and dried under vacuum. The linear and cyclic carbonate content was determined. The isolated polymer was redissolved in 50ml dry DMSO and 25ml dry pyridine. 2.5 equivalents 2-S-trityl mercapto ethylamine (1), compared to the determined total carbonate content, were added and the reaction was allowed to continue for 48 hours. The polymer was isolated by precipitation in 200ml ethanol and 200ml ether. After filtration and washing with ethanol and ether, the polymer was further purified by preparative GPC on Sephadex G25 (eluent: double distilled water), followed by freeze drying.

The product was analysed by NMR spectroscopy, and from the integrated trityl proton at δ = 7.2-7.4 and the integrated dextran C_1 proton at δ = 4.5 the degree of dextran substitution was calculated as 3.5%.

c) <u>2-S-(methoxythiocarbonato)ethylaminocarbamoyl dextran (3)</u>

0.5g of product (2) was dissolved in 5ml dry formamide and 650µl dry methanol. 500µl (5.5. mmol) methoxycarbonyl sulphenyl chloride, prepared as described by Galpin and Hoyland, was added and the reaction was allowed to continue for 20 hours. The polymer was isolated by precipitation in 60ml ether and 60ml methanol. After filtration and washing with methanol and ether, the polymer was further purified by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

NMR (360 MHz, D_2O)	: C-1 H dextran	δ = 4.95 ppm
	CH3-O-CO-S-	δ = 3.85 ppm
	aromatic H's no longer visible	

d) <u>2-(isopropyldithio)ethylaminocarbamoyl dextran (4)</u>

To demonstrate coupling to a model thiol, 0.1g of product (3) was dissolved in 5ml phosphate buffer (pH=7, 0.1M) and 100µl (1.1 mmol) 2-propanethiol was added. The coupled polymer was isolated after 1 hour reaction by precipitation in 30ml ether and 30ml methanol. After filtration and washing with methanol and ether, the coupled polymer was further purified by preparative GPC on Sephadex G25 (eluent: double distilled water), followed by freeze drying. The polymer/isopropyl product was also analysed by NMR spectroscopy.

NMR (360 MHz, D_2O)	: C-1 H dextran	δ = 5.05 ppm
	CH3 isopropyl	δ = 1.32 ppm

e) 2-(glutathionyldithio)ethylaminocarbamoyl dextran (5)
To demonstrate coupling to a model thiol-containing bioactive peptide or protein, 0.15g of product (3) was dissolved in 15ml oxygen free phosphate buffer (pH=7, 0.1M) and reacted for 1 hour with 0.46g (1.50 mmol) reduced glutathione. The coupled polymer was isolated by precipitation in 60ml ether and 60ml methanol, followed by centrifugation. The isolated product was further purified by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

NMR (360 MHz, D_2O)	:	C-1 H dextran	δ = 5.2 ppm
		NH-CO-CH2- (glu)	δ = 2.18 ppm
		NH-CO-CH2-CH2(glu)	δ = 2.52 ppm
		S-S-CH2 (cys)	δ = 2.95 ppm

f) Coupling of 2-hydroxy-propylamine to product (2) in order to synthesise an amine-coupled compound (6)

This example is included to show the manner in which a multi-functional polymer may be coupled to other molecular entities such as drug molecules via non-thiol groups prior to coupling with a thiol-containing compound via a reactive sulphenyl thiocarbonate group.

0.5g of product (2) was dissolved in 15ml dry DMSO and 15ml dry pyridine and cooled to 0°C. 170mg (0.84 mmol) 4-nitrophenyl chloroformate and 12mg (0.1 mmol) 4-dimethylamino pyridine were added and the reaction was allowed to continue for 4 hours at 0°C. The polymer was precipitated in 100ml ether and 100ml ethanol, filtered and washed with methanol and ether. The isolated product was dried under vacuum.

The polymer was redissolved in 20ml dry DMSO and 10ml dry pyridine and reacted with 162µl (2.1 mmol)

(±)-1-amino-2-propanol for 48 hours. This was chosen as a model amine to demonstrate the possible coupling to an amine-containing drug molecule whilst retaining the protected thiol group for subsequent activation and coupling to another, thiol-containing, compound. The polymer was precipitated in 100ml ether and 100ml ethanol, filtered and washed with methanol and ether. The product (6) was further purified by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

NMR (360 MHz, DMSO) :	C-1 H dextran	δ = 4.4 ppm
	<u>CH3</u>-CH(OH)-	δ = 1.02 ppm
	aromatic H's	δ = 7.3 ppm

(g) <u>The reaction of product (6) with methoxycarbonyl sulphenylchloride to synthesise an activated disulphide derivative - product (7)</u>

0.15g of product (6) was dissolved in 1.5ml dry formamide and 200µl dry methanol and 150µl (1.66 mmol) methoxycarbonyl sulphenylchloride was added. The reaction was allowed to continue for 20 hours. The polymer was precipitated in 30ml ether and 30ml methanol, isolated by centrifugation and further purified by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

NMR analysis indicates that all aromatic groups were replaced

(h) <u>The reaction of product (7) with 2-propanethiol; the synthesis of product (8)</u>

To demonstrate coupling to a model thiol, 50mg of product (7) was dissolved in 2.5ml phosphate buffer

(pH=7, 0.1M) and reacted for 1 hour with 50µl (0.54 mmol)· 2-propanethiol. The coupled polymer was precipitated in 30ml ether and 30ml methanol, isolated by centrifugation and further purified by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

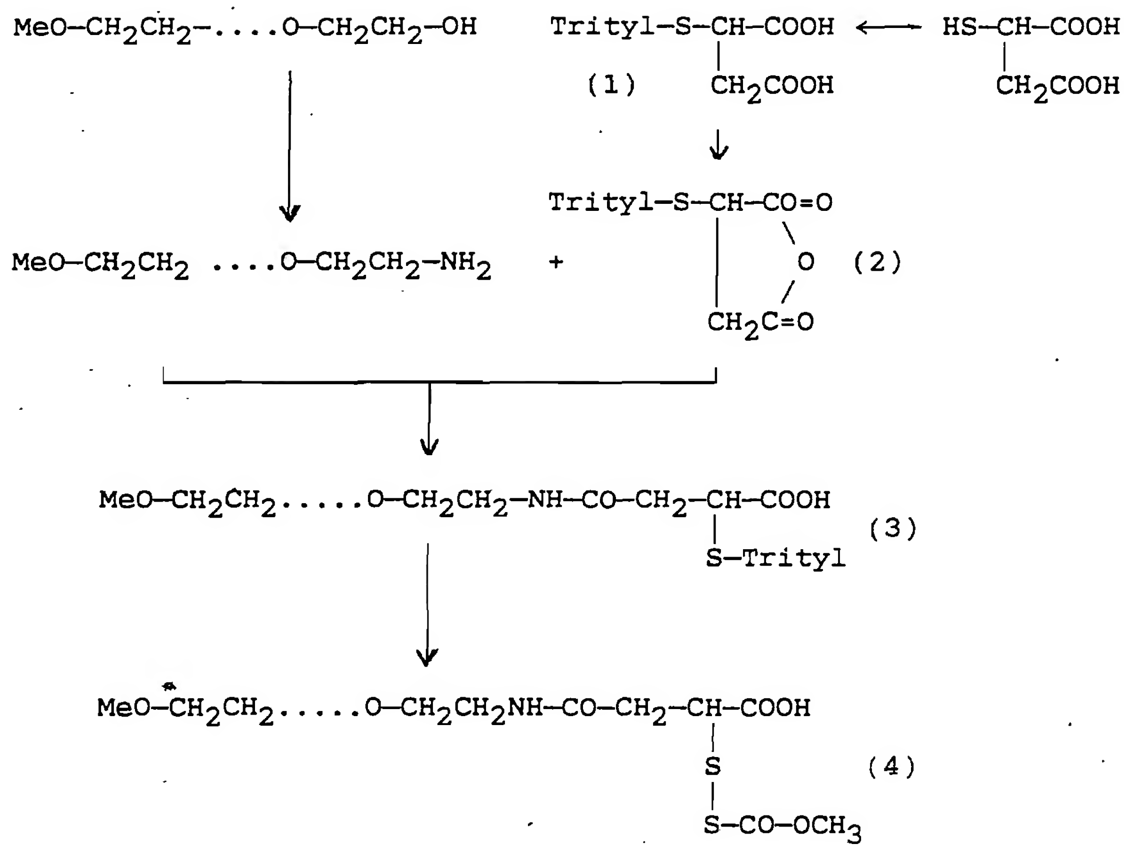
NMR (D_2O)	: C-1 H dextran	δ = 5.0	ppm
	<u>CH3</u>-CH(OH)-	δ = 1.15	ppm
	S-S-CH(<u>CH3</u>)2	δ = 1.3	ppm

Thus, this example demonstrates the feasibility of the chemistry for selective successive coupling of both amino-containing molecules, e.g. a drug, and thiol-containing molecules, e.g. a protein, to a multi-functional polymer such as dextran.

<u>EXAMPLE 3</u>

<u>α-methoxy-Ω-(S-methoxythiocarbonyl)mercaptosuccinylamino poly(oxyethylene) (4)</u>

This is an example of an alternative approach for the preparation of a reactive sulphenyl thiocarbonate derivative of a polymer such as polyethylene glycol (PEG) in which a thiol or protected thiol for conversion into a reactive terminal group is introduced by way of a compound which becomes incorporated as an end unit of the polymer chain. The reaction scheme may be depicted as follows:



<u>Experimental Part</u>

Materials and instruments: All products were used without further purification. Methanol was dried over magnesium and all other solvents were dried over calcium hydride. All ^{1}H NMR spectra were run on a Bruker WH360 or a Bruker WH500 spectrometer. IR spectra were recorded on a Beckman 4230 spectrometer. Preparative GPC was carried out on a Pharmacia column (K2670), packed with Sephadex G15 (Pharmacia: Sweden). Double distilled water was used as eluent (2ml/ml). Eluting components were detected via refractive index.

(a) <u>S-trityl mercapto succinic acid (1)</u>

10g (66.7 mmol) mercapto succinic acid and 17g (61 mmol) triphenylmethyl chloride were dissolved in 100ml THF. The solution was refluxed for 3 hours under nitrogen. The solvent was removed under reduced pressure. The residue

was dissolved in ethyl acetate and extracted 3 times with 100ml water. The pooled ethyl acetate was dried over $MgSO_4$. The $MgSO_4$ was removed by filtration and the solution was evaporated under reduced pressure. The residue was recrystallized from ethyl acetate/hexane. The filtrate was concentrated to 1/3 of its original volume and cooled to −18°C. The second crop was collected by filtration and the pooled crystals were dried under vacuum.

^1H NMR spectrum (500 MHz, solvent $CDCl_3$):
the ABX system: δ_A=1.51 ppm, m=2x2, J_{A-X}=3.5Hz, J_{A-B}=17.8Hz (1H); δ_B=2.48 ppm, m=2x2, J_{B-X}=11.5Hz, J_{B-A}=17.8Hz (1H); δ_X=3.26 ppm, m=2x2, J_{X-A}=3.5Hz, J_{X-B}=11.5Hz (1H);
the aromatic para protons: δ=7.24 ppm, m (3H);
the aromatic meta protons: δ=7.31 ppm, m (6H);
the aromatic ortho protons: δ=7.52 ppm, m (6H)

IR spectrum (KBr):
intensive OH vibration between 3400 and 2500cm^{-1};
C=O stretching vibration of acid functions: v=1700cm^{-1};
aromatic vibrations: v=1610cm^{-1}, v=1590cm^{-1}, v=1490cm^{-1}

(b) S-trityl mercaptosuccinic anhydride (2)

4.5g (11.5 mmol) S-trityl mercaptosuccinic acid was added to a solution of 2.36g (11.5 mmol) DCC in 15ml CH_3CN. The solution was stirred overnight. The precipitated DCU was removed by filtration and washed with CH_3CN. The filtrate was evaporated under reduced pressure and the residue was dried under vacuum.

^1H NMR spectrum (360 MHz, solvent $CDCl_3$):
the ABX system: δ_A=2.10 ppm, m=2x2, J_{A-B}=19.5Hz, J_{A-X}=10.1Hz (1H); δ_B=2.35 ppm, m=2x2, J_{B-A}=19.5Hz, J_{B-X}=6.6Hz (1H); δ_X=3.70 ppm, m=2x2, J_{X-A}=10.1Hz, J_{X-B}=6.6Hz (1H);

the aromatic para protons: δ=7.28 ppm, m (3H);
the aromatic meta protons: δ=7.35 ppm, m (6H);
the aromatic ortho protons: δ=7.51 ppm, m (6H).

IR spectrum (KBr):
C-H stretching vibrations: $v=2850cm^{-1}$, $v=2920cm^{-1}$, $v=3020cm^{-1}$, $v=3050cm^{-1}$;
C=O vibrations of the anhydride; $v=1855cm^{-1}$, $v=1770cm^{-1}$;
aromatic vibrations: $v=1590cm^{-1}$, $v=1575cm^{-1}$, $v=1485cm^{-1}$.

(c) <u>α-methoxy-Ω-(S-trityl)mercaptosuccinylamido-poly(oxyethylene) (3)</u>

0.5g (0.667 mmol) amino terminated poly(oxyethylene) and 0.25g (0.667 mmol) S-trityl mercaptosuccinic anhydride (2) were dissolved in 5ml CH_2Cl_2. The reaction was allowed to continue overnight. The solvent was removed under reduced pressure and the residue was dried under vacuum. The polymer was isolated by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

^1H NMR spectrum (360 MHz, solvent: MeOD):
the ABX-system: isomer 1: A-proton: δ=1.56 ppm, m=2x2, J_{A-B}=17.0Hz, J_{A-X}=5.0Hz;
B-proton: δ=2.42 ppm, m=2x2 , J_{B-A}=17.0Hz, J_{B-X}=11.10Hz;
X-proton: shifted under the polymer peak;
isomer 2: A-proton: δ=1.99 ppm, m=2x2, J_{A-B}=17.0Hz, J_{A-X}=4.5Hz;
B-proton: δ=2.59 ppm, m=2x2, J_{B-A}=17.0Hz, J_{B-X}=10.0Hz;
X-proton: shifted under the polymer peak;
methoxy group of poly(oxyethylene): δ=3.35 ppm, m=1 (3H);
CH α of the amide group: δ=3.2 ppm, m (2H);

CH_2 of the amide group: δ=3.4 ppm, m (2H);
CH_2 groups of poly(oxyethylene): δ=3.65 ppm;
para protons of the trityl group: δ=7.23 ppm, m (3H);
meta protons of the trityl group: δ=7.30 ppm, m (6H);
ortho protons of the trityl group: δ=7.48 ppm, m(6H);
ratio least hindered isomer/most hindered isomer equal to 2/1.

IR spectrum (film):
C–O–C stretching vibration: ν=1100cm^{-1};
the amide stretching vibration: ν=1660cm^{-1};
the acid stretching vibration: ν=1725cm^{-1};
the C–H stretching vibrations: ν=2860cm^{-1}, ν=3020cm^{-1}, ν=3040cm^{-1};
the aromatic vibrations: ν=1590cm^{-1}.

Chemical analysis: ninhydrin negative

(d) <u>α–methoxy–Ω–(S–methoxythiocarbonyl)mercapto succinylamido poly(oxyethylene) (4)</u>

230mg (0.18 mmol) α–methoxy–Ω–(S–trityl)mercapto succinylamido poly(oxyethylene) (product 3) was dissolved in 1.5ml CH_2Cl_2 and 1.5ml MeOH. 70µl carbomethoxycarbonyl sulphenyl chloride was added. The reaction was allowed to continue for 2 hours. The solvent was removed under reduced pressure and the polymer was isolated by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

^{1}H NMR spectrum (360 MHz, solvent: $CDCl_3$):
ABX system: isomer 1: δ=2.64 ppm, m=2x2, J_{A-B}=15.6Hz, J_{A-X}=5.8Hz; δ_B=2.9 ppm, m=2x2, J_{A-B}=15.6Hz, J_{B-X}=9.0Hz; δ_X=3.95 ppm, J_{A-X}=5.8Hz, J_{B-X}=9.0Hz;
isomer 2: δ_A=2.75 ppm, J_{A-X}=5.7Hz, J_{A-B}=15.6Hz; δ_B=302 ppm, J_{A-B}=15.6Hz, J_{B-X}=8.9Hz; δ_X=4.15 ppm,

J_{A-X}=5.7Hz, J_{B-X}=8.8Hz;
isomer 3: δ_A=2.73 ppm, J_{A-X}=5.6Hz, J_{A-B}=17.1Hz; δ=3.1 ppm, J_{A-B}=17.0Hz, J_B-X=8.7Hz; δ_X=4.02 ppm, J_{A-X}=5.6Hz, J_{B-X}=8.7Hz;
CH_3 of the methylether: δ=3.35 ppm, m=1 (3H);
CH_2 α of the amide: δ=3.43 ppm, broadened (2H);
CH_2 groups of the poly(oxyethylene): δ=3.65 ppm;
CH_3 of the methylester: δ=3.9 ppm (3H);
NH: δ=6.65 ppm

IR spectrum (film):
the amide stretching vibration: ν=1660cm^{-1};
the acid stretching vibration: ν=1705cm^{-1};
the ester stretching vibration ν=1730cm^{-1};
the C-H stretching vibrations: ν=2860cm^{-1}.

(e) <u>α-methoxy-Ω-(isopropyldithio)succinylamido poly-(oxyethylene) (5)</u>

To demonstrate coupling of product (4) to a thiol compound, 200mg (0.206 mmol) of the α-methoxy-Ω-(S-methoxy thiocarbonyl)mercapto succinylamido poly(oxyethylene) (4) and 13µl (0.21 mmol) isopropylthiol were dissolved in 2ml phosphate buffer pH=7. The reaction was allowed to continue for 3 hours. The end coupled polymer was isolated by preparative GPC on Sephadex G15 (eluent: double distilled water), followed by freeze drying.

^{1}H NMR spectrum (360 MHz, solvent $CDCl_3$) :
CH_3 of the isopropylthiol: δ=1.31 ppm, m (6H);
ABX-system of the isomers and CH of the isopropyl group: several m between δ=2.55 ppm and δ=3.15 ppm (3H);
CH_3 of the methylether: δ=3.37 ppm, m=1;
CH_2 groups of the poly(oxyethylene): δ=3.65 ppm, broadened;
CH_2 α of the amide: δ=3.43ppm, m (2H)

IR spectrum (film):

the C–O–C stretching vibration: v–1090cm^{-1};

the amide stretching vibration: v=1650cm^{-1};

the acid stretching vibration C=O vibration: v=1725cm^{-1};

the C–H stretching vibrations: v=2870cm^{-1}

EXAMPLE 4

A. Synthesis of a poly–N–(2–hydroxyethyl)–L–glutamine with a thiol-specific reactive end group

This is an example of the synthesis of a polyamino acid with a reactive sulphenyl thiocarbonate end group using a mercaptoamine derivative with a protected thiol group, e.g. 2-S-(tert. butyl)mercaptoethylamine tBu-S-CH_2-CH_2-NH_2 or the equivalent trityl protected derivative trityl-S-CH_2-CH_2-NH_2, to initiate polymerisation of an N-carboxyanhydride (NCA) monomer, the mercaptoamine derivative becoming incorporated as an end unit in the polymer chain and serving also to introduce a thiol group for conversion into the reactive sulphenyl thiocarbonate.

Experimental part

a) Preparation of gamma–benzyl–L–glutamate (1)

50 ml 98% H_2SO_4 was added to a suspension of 500 ml of diethyl ether and 500 ml benzyl alcohol. Ether was removed under reduced pressure. 73.5g of L–glutamic acid (0.5 mol) was added. After 20 hours stirring at room temperature, 900 ml of H_2O was added while cooling to 10°C. The pH was brought up to 6-6.5, with LiOH, to precipitate the gamma–benzyl–L–glutamate.

The precipitate was left standing at 0°C for 1 hour. The ester was isolated by filtration and recrystallized with H_2O. Characterization of the gamma-ester was done using ^1H-NMR spectroscopy, TGA; at 165°C cyclization occurs removing the benzyl alcohol. DSC showed a melting point of 183°C.

b) <u>Synthesis of the N-carboxyanhydride of gamma-benzyl-L- glutamate (NCA) (2)</u>

20g of gamma-benzyl-L-glutamate (1) (84 mmol) was dissolved in 200 ml of dry THF. 8.34g of triphosgene (28 mmol) was added very carefully while the mixture was held at a temperature of 50°C. After 15 minutes the solution cleared. After 3 hours N_2 was bubbled through the solution for 2 hours. The NCA's obtained were precipitated in a 5 fold excess of dry hexane. After filtration, the product was dissolved in a minimal amount of ethyl acetate and recrystallized in a large amount of hexane compared to the amount of ethyl acetate. Recrystallization was performed twice.

The NCA was then dried at room temperature, under reduced pressure.

The I.R.-spectrum showed the typical absorption signals for the gamma-ester: 1725 cm^{-1} and the anhydride: 1790 cm^{-1} 1850 cm^{-1}.

c) <u>Preparation of poly-gamma-benzyl-L-glutamate carrying a potential thiol specific end group. (t.But-PBLG).</u>

6g NCA (2) (22.8 mmol) was dissolved in 250 ml dry 1,4-dioxane. The selected degree of polymerisation (DP) was obtained by choosing the amount of the initiator (I) used, in this case 2-S-(tert. butyl)mercapto ethylamine. DP=(A)/(I).

The reaction was followed by I.R. spectroscopy. After total conversion (no NCA's left), the polymer was precipitated in a 5-fold excess of dry ether. Recrystallization was performed by dissolving the polymer in $CHCl_3$ and precipitating in ether. The DP could be calculated from ^1H NMR data. The DP obtained was 14 in this case.

d) <u>Preparation of poly-N-(2-hydroxyethyl)-L-glutamine. (t.But-PHEG).</u>

3g of the t.But-PBLG (13 mmol benzyl esters) was dissolved in 10 ml DMF. 150 mg 2-hydroxypyridine (2.6 mmol) and 20 ml 2-amino-ethanol (260 mmol) was added. After 55 hours of stirring at room temperature, the polymer was precipitated in a five-fold excess of $CHCl_3/Et_2O$:50/50.

The product was purified using a strong acidic ion exchanger, Amberlite c.g.200, and lyophilised.

e) <u>Preparation of a sulphenylthiocarbonate substituted polyhydroxyethyl-L-glutamine</u>

500 mg of the t.But-PHEG (156 mmol) was dissolved in 15ml DMF/1.2ml MeOH and stirred for 4 hours with 785 µl methoxycarbonylsulphenylchloride (8.68 mmol).

The pure polymer was obtained by precipitating it in a 5-fold excess of $CHCl_3/Et_2O$:50:50 and using a preparative GPC column filled with TSK-HW40(S).

B. <u>Coupling of poly-N-(2-hydroxyethyl)-L-glutamine with B-chain of insulin</u>

By way of example of coupling of the sulphenyl-thiocarbonate substituted polyamino acid compound,

synthesised as described above, with a thiol-containing compound, coupling was demonstrated with an insulin B-chain protein which includes a plurality of thiol groups.

a) Preparation of Insulin B-chain

250 mg insulin (41.7 µmmol) was mixed with 18.05 g recrystallized urea and dissolved in 15 ml 1.44 M tris-buffer, 0.013 M EDTA, pH 8.6. The volume was made up to 37.5 ml with water, and 0.5 ml mercaptoethanol was added under N_2. The mixture was then brought to 60 ml with 8 M urea, 0.2% EDTA, and sealed at 20°C for 4 hours.

b) Separation of insulin A-chain from insulin B-chain

The separation was performed under an atmosphere of Argon to avoid re-oxidation.

The column used was a weakly acidic cation exchanger (Amberlite CG—50 supplied by Sigma). The column dimensions were 2.5 x 2.5 cm. First, insulin A-chain was eluted with 500ml 5% acetic acid. Insulin B-chain was then eluted with 210 ml 50% acetic acid. The yield of insulin B-chain by this process was approximately 85 mg (24.3 µmol) containing 48.6 mol thiol groups.

c) Coupling of insulin B-chain to poly-HEG

The insulin B-chain, together with the acetic acid was immediately collected in a flask containing 48.6 µmol of the sulphenylthiocarbonate substituted poly-HEG, dissolved in 30 ml oxygen-free phosphate buffer, pH 7, under Argon atmosphere. This mixture was left to stand at room temperature for two hours. The volume

was rotary evaporated down to 15ml. The solution was then dialysed for 12 hours. (dialysis tubing: MWCO-1000 supplied by Polylab.)
The reaction product was analysed by analytical HPLC. Preparative HPLC on C-18 phase allowed isolation of the desired coupling product. The chemical structure of the latter was confirmed as being a 1:2 conjugate (1 B-chain, 2 poly-HEG) by reduction with dithiotreitol and HPLC and capillary electrophoresis analysis of the products formed.

This therefore provided an example of a protein being conjugated with more than one polymer molecule. Conjugates of this kind may be particularly useful for modifying the hydrophilic/hydrophophic characteristics and/or immunogenicity of proteins in biological systems, as previously indicated.

Additional Notes

1. S–Trityl cysteine has also been used to introduce S–trityl groups along a dextran chain or at the end of a monomethoxypolyethyleneglycol chain. The chemistry is similar to that herein described using 2–S–trityl-mercaptoethylamine, Trit–S–CH_2CH_2–NH_2.

2. All examples presented above include the use of a trityl or tertiary butyl HS–protecting group. As previously indicated, however, an acetamidomethyl group CH_3CO–NH–CH_2– may be used as an alternative. The latter is known as a protective group for –SH in cysteine (see P. Marbach, J. Rudinger: Helv. Chim. Acta 57, 403, (1974)).

Thus, S–acetamidomethyl–L–cysteine (and also the methyl ester of S–acetamidomethyl–L–cysteine)

$$CH_3CO{-}NH{-}CH_2{-}S{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH(Me)$$

has been attached to polyethylene–glycol and dextran in a similar way to that described for the S–trityl mercapto ethylamine.

3. In addition to the specified use of $HS{-}CH_2CH_2{-}NH_2$, cysteine, and cysteine methyl ester, as thiol-containing amines for introducing thiol or protected thiol groups into the polymers, obviously other aminothiols could be used in a similar way.

The invention also includes all novel and inventive features and aspects herein disclosed, either explicitly or implicitly and either singly or in combination with one another, and the scope of the invention is not to be construed as being limited by the illustrative examples or by the terms and expressions used herein merely in a descriptive or explanatory sense.

CLAIMS

1. A process for preparing a conjugate compound in which a macromolecular chain polymer is selectively coupled to another organic molecular entity, said process being characterised by the steps of:

(a) modifying or forming the polymer so as to provide it with a thiol or protected thiol group in a predetermined position or positions,

(b) providing a derivative of said other molecular entity containing a thiol or protected thiol group,

(c) treating the compound of either step (a) or step (b) to convert the thiol or protected thiol group thereof into a thiol-specific reactive disulphide or sulphenyl thiocarbonate group, —S—S—CO—OR where R is alkyl, aryl or alkylaryl, and

(d) reacting together the compound of step (a) or step (b) that is not utilised in step (c) with the product of step (c) whereby the thiol-specific reactive disulphide or sulphenyl thiocarbonate group of the latter reacts with the thiol or protected thiol group of the other reactant to result in the formation of an unsymmetrical disulphide covalent linkage between the polymer and said other molecular entity.

2. A process as claimed in Claim 1, wherein steps (a) and (c) are carried out in organic media and step (d) is carried out, without application of heat, in aqueous media.

3. A process as claimed in Claim 1 or 2 wherein step (c) is carried out by treating the polymer compound of step (a), thereby to form the thiol-specific reactive disulphide or sulphenyl thiocarbonate group or groups on the polymer.

4. A process as claimed in Claim 3 wherein a said thiol-specific reactive disulphide or sulphenyl thiocarbonate group is formed at an end or ends of the polymer chain.

5. A process as claimed in Claim 3 wherein there is only a single said thiol-specific reactive disulphide or thiocarbonate group formed on the polymer chain.

6. A process as claimed in Claim 5 wherein the single thiol-specific reactive disulphide or thiocarbonate group is an end terminal group.

7. A process as claimed in any of the preceding claims wherein the polymer is a polyethyleneglycol or polyethylene oxide polymer.

8. A process as claimed in Claim 3 wherein a said thiol-specific reactive disulphide or sulphenyl thiocarbonate group is formed on one or more side groups of the polymer chain.

9. A process for preparing a conjugate compound as claimed in any of the preceding claims wherein the polymer is initially a pre-existing molecule that contains only one or more end or side chain functional groups which are not sulphur containing groups, further characterised in that during step (a) the polymer is first treated under controlled conditions with an activating agent to convert said existing functional group or groups, or a certain proportion thereof, into a reactive form that is adapted more readily to link with a mercaptoamine derivative or other carrier molecule that is then used to introduce the thiol or protected thiol group(s).

10. A process as claimed in Claim 9 wherein the polymer is dextran.

11. A process for preparing a conjugate compound as claimed in any of Claim 3 to 7, wherein the thiol or protected thiol group is introduced into the polymer by way of a carrier molecule that becomes incorporated as an end unit of the polymer chain, said carrier molecule comprising

a compound containing the thiol or protected thiol group, and said thiol or protected thiol group is converted subsequently into the required thiol-specific reactive disulphide or sulphenyl thiocarbonate group for establishing an end terminal coupling to the thiol derivative of said other molecular entity.

12. A process as claimed in Claim 11 in which the compound providing the carrier molecule used to introduce the thiol or protected thiol group is an anhydride of mercapto-succinic acid.

13. A process as claimed in Claim 11 in which the polymer is a polyamino acid synthesised from monomer units provided by an amino acid N-carboxyanhydride (NCA) derivative or derivatives, and the compound providing the carrier molecule which introduces the thiol or protected thiol group is a mercaptoamine derivative which also initiates the polymerization of the NCA monomer units.

14. A process as claimed in any of the preceding claims wherein step (a) is carried out so as to provide the polymer with a protected thiol group in one or more predetermined positions, further characterised in that, intermediate steps (a) and (c), the polymer is treated so as to couple a plurality of non-sulphur-containing functional groups thereof with drug molecules through biodegradable covalent linkages other than disulphide bonds.

15. A process for preparing a conjugate compound in which a synthetic macromolecular chain polymer is selectively coupled to another organic molecular entity, characterised in that during the process

(a) the polymer is synthesised from monomer units in a polymerisation reaction initiated by a mercaptoamine derivative that becomes incorporated as an end unit of the polymer chain;

(b) the polymer is formed with a thiol-specific reactive disulphide or sulphenyl thiocarbonate group, —S—S—CO—OR where R is alkyl, aryl or alkylaryl, in said end unit of the polymer chain;

(c) the polymer containing said thiol-specific reactive disulphide or sulphenyl thiocarbonate group is reacted with a thiol group containing derivative of said other molecular entity whereby the thiol-specific reactive disulphide or sulphenyl thiocarbonate group of the polymer reacts with the thiol group of the other reactant resulting in the formation of an unsymmetrical disulphide covalent linkage between the polymer and said other molecular entity.

16. A process as claimed in Claim 15 wherein step (c) is carried out without application of heat in aqueous media after carrying out preceding steps in organic media.

17. A process as claimed in Claim 15 or 16 wherein the polymer is a synthetic polyamino acid which prior to step (c) is formed with a single said thiol-specific reactive group in a terminal position at one end of the polymer chain for endwise coupling to the thiol derivative of the said other molecular entity.

18. A process as claimed in Claim 17 wherein the polyamino acid is synthesised by polymerisation of an amino acid N-carboxyanhydride (NCA) derivative or derivatives.

19. A process as claimed in any of Claims 15 to 18 wherein the mercaptoamine derivative used to initiate the polymerisation has a thiol group thereof activated and converted into the required thiol-specific reactive disulphide or sulphenyl thiocarbonate group prior to its use for initiating the polymerisation.

20. A process as claimed in any of Claims 15 to 18 wherein the mercaptoamine derivative used to initiate the

polymerisation has a protected thiol group which is activated and converted into the required thiol-specific reactive disulphide or sulphenyl thiocarbonate group only after the polymer is produced and immediately prior to step (c).

21. A process as claimed in any of Claims 15 to 20, wherein the polymer produced is also formed with a plurality of hydroxyl or other functional groups on side chains or spacers, available for coupling to drugs or other additional molecular entities, along the length of the chain.

22. A process as claimed in Claim 21 wherein the polymer is treated so as to couple drug or other bioactive molecules through covalent linkages to said hydroxyl or other functional groups prior to forming the thiol-specific reactive disulphide or sulphenyl thiocarbonate group of step (b).

23. A process as claimed in any of the preceding claims wherein the thiol-containing other molecular entity that is coupled to the polymer is a thiol-containing protein.

24. A process as claimed in any of Claims 1 to 23 wherein the thiol-containing other molecular entity that is coupled to the polymer is an antibody or antigen binding fragment thereof.

25. A process as claimed in any of Claims 1 to 22 wherein the or each thiol-containing other molecular entity that is coupled to the polymer is a thiol-containing drug molecule.

26. A method of selectively coupling a thiol-containing protein or polypeptide to a macromolecular polymer carrier wherein the polymer carrier is provided or formed with a single thiol-specific reactive sulphenyl thiocarbonate group and is then reacted with the protein or polypeptide

so as to result in interaction with a thiol group in the latter and fragmentation of the sulphenyl thiocarbonate group, with establishment of a covalent disulphide linkage between the protein or polypeptide and the macromolecular polymer carrier to form a conjugate compound thereof.

27. A method as claimed in Claim 26 wherein the single thiol-specific reactive thiocarbonate group is an end terminal group.

28. A conjugate compound prepared by the process claimed in any of the preceding claims and adapted for therapeutic or diagnostic use in biological systems.

29. A conjugate compound as claimed in Claim 28 adapted for use as a targeting drug delivery agent.

30. A conjugate compound as claimed in Claim 28 or 29 wherein the polymer is coupled to a site-specific targeting moiety.

31. A conjugate compound as claimed in any of Claims 28 to 30 wherein the polymer is coupled to a protein.

32. A conjugate compound as claimed in Claim 31 wherein the protein is an antibody or antigen binding fragment thereof.

33. A conjugate compound as claimed in Claim 31 or 32 wherein the polymer is coupled to the protein in a 1:1 ratio.

34. A conjugate compound as claimed in any of Claims 30 to 33, wherein the polymer is also coupled to a plurality of bioactive drug molecules.

35. A pharmaceutical formulation containing a conjugate compound as claimed in any of Claims 28 to 34.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/00515

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 47/48 C 08 F 4/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
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Y		1,3,7,9,10-13,15-27

X	EP-A-0 313 873 (AMERICAN CYANAMID CO.) 3 May 1989, see page 3, table 2; page 6, line 24 - page 7, line 53	1-6,8,9,11,23-25,28-35

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° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18-07-1991	16 SEP 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

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Y	FILE SERVER STN, FILE BIOSIS, abstract no. 90:161257 & Biological Abstract 89:88675 & Int. J. PHARM., (Amst.) 57(3), 1989, 263-272, see abstract	1,9,10
Y	J. AM. CHEM. SOC., vol. 92, 1970, (Washington, D.C., US); S.J. BROIS et al.: "A new pathway to unsymmetrical disulfides. The thiol-induced fragmentation of sulfenyl thiocarbonates", pages 7629-7631, see the whole article (cited in the application)	26,27
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The members are as contained in the European Patent Office EDP file on 10/09/91
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82